IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of :

Kristof CHWALISZ et al. Group Art Unit: 1617

Serial No.: 10/043,232 Examiner: TAVERS, Russell S.

Filed: January 14, 2002

For: IMPLANTATION RATES AFTER IN VITRO FERTILIZATION, AND TREATMENT OF INFERTILITY AND EARLY PREGNANCY LOSS WITH A NITRIC OXIDE DONOR OR SUBSTRATE ALONE OR IN COMBINATION WITH PROGESTERONE, AND A METHOD FOR CONTRACEPTION WITH NITRIC OXIDE INHIBITORS IN COMBINATION WITH ANTIPROGESTINS OR OTHER AGENTS

DECLARATION UNDER 37 C.F.R. §1.132

I, Dr. Robert E Garfield, being duly warned, declare that:

I am a citizen of the United States, residing in Texas.

I am one of the inventors of the above-captioned application and am familiar with the invention described therein and with the outstanding grounds for rejection made against the claims of the application.

My expertise to make this declaration is demonstrated in the attached CV. I have a financial interest in this application and any patent which issues from it.

The following tests were either performed by me or conducted under my supervision:

Materials and methods

Animals

Early pregnant female Wistar rats (Schering, TZH, Berlin, Germany) were used for all experiments. These animals were about 300 g each. The animals were kept under standard conditions. The light/dark-cycle was 14/10 h (light 6:30-20:30). The presence of sperm in the vaginal smear on the morning following mating was defined as day 1 post coitum (day I p.c.).

Compounds and formulations

L-NAME (a NOS inhibitor) (Sigma-Aldrich Chemie, Munich, Germany) was dissolved in sterile 0.9% saline solution. Osmotic minipumps (Alza, Palo Alto, CA, USA; model 2MLI with a pumping rate of 10 µl/h) were filled with vehicle or with L-NAME solution and implanted s.c. during ether anaesthesia. The drug-release was verified by opening the minipumps during autopsy to determine the remaining volume. Aminoguanidine (a NOS inhibitor) (Sigma-Aldrich Chemie) was dissolved in water at pH 6.0 and administered orally (p.o.) in 1 ml. The specific progesterone antagonist (i.e., antiprogestin) onapristone (11(3-[4-(dimethylamino)phenyl]-17α-hydroxy-17β-(3-hydroxypropyl)-13 (α-estra-4,9-dien-3-one) was formulated in 0.2 ml benzylbenzoate + castor oil (I + 4 vol/vol) and administered s.c.

Experimental design

Treatments with L-NAME (50 mg/rat/day s.c.) and aminoguanidine (120 mg/rat/day) alone as well as in combination with low-dose onapristone (0.3 mg/rat/day s.c.) were performed after randomization in accordance with two experimental protocols (Figure IA, B). L-NAME at this dose produced hypertension and fetal growth restriction in late pregnant rats (Liao *et al.*, 1997).

The effects of NO inhibition and low-dose onapristone on implantation after treatment during the preimplantation phase (days 1-4 p.c.)

Experiment 1: the effects of L-NAME in the presence and absence of onapristone on implantation after treatment on days 1-4 p.c. Osmotic minipumps containing L-NAME or 0.9% saline (vehicle) were implanted s.c. on day I p.c. and removed on day 4 p.c. Onapristone or the respective vehicle (controls) was administered (s.c.) once daily on days 1-4 p.c. *(n* = 7/group). Group 1 (vehicle control) received the respective vehicles (saline-containing mini-pump and onapristone vehicle). Group 2 was treated with L-NAME and the onapristone vehicle. Group 3 received onapristone and the L-NAME vehicle (i.e. saline-containing mini-pump was implanted). Group 4 received a combination of L-NAME plus onapristone. Peripheral blood for progesterone and oestradiol radioimmunoassays was collected from the retro-orbital plexus during CO_2 anaesthesia at 9-10 a.m. on days 1, 5, 7 and 9 p.c. During autopsy on day 9 p.c. the uteri were opened, photographed and macroscopically examined for implantation sites and resorptions. They were then removed and processed for histology.

Experiment 2: the effects of aminoguanidine in the presence and absence of onapristone on implantation after treatment on days 1-4 p.c. Treatments were performed as described for experiment 1, using the same animal numbers per group. Group 1 (controls) was treated orally and s.c. with both vehicles. Group 2 received aminoguanidine orally and onapristone vehicle s.c. Group 3 was treated s.c. with onapristone and with the aminoguanidine vehicle orally. Group 4 was treated orally with aminoguanidine and s.c. with onapristone at the respective dose. Peripheral blood collections and autopsy (day 9 p.c.) were performed as described for experiment 1.

Experiment 3: pregnancy outcome (on day 19 p.c). after treatment with L-NAME and aminoguanidine in the presence and absence of onapristone on days 1-4 p.c. Treatments were performed as described for experiments 1 and 2. However, in order to assess the effects of treatment on pregnancy outcome, the autopsy was performed on day 19 p.c., i.e. approximately 3 days before birth (term: days 21-22 p.c.). During autopsy, the number and condition of pups, fetal weight, placental weight and the number of resorptions per uterus were recorded. In addition, the empty uteri were stained with 10% ammonium sulphide in order to identify early resorptions.

The effects of NO inhibition and low-dose onapristone on implantation after treatment during the peri- implantation phase (days 5-8 p.c.)

Experiment 5: the effects of L-NAME alone or in combination with low-dose onapristone after treatment on days 5-8 p.c. Pregnant rats *(n* = 8/group) were treated on days 5-8 p.c. using the same groups as described for experiment 1.

Experiment 6: the effects of aminoguanidine alone and in combination with low-dose onapristone after treatment on days 5-8 p.c. Rats were randomly allocated to four groups *(n* = 7/group)

and were treated on day 5-8 p.c. The same treatment groups as described for experiment 2 were used.

Experiment 7: pregnancy outcome (on day 19 p.c). after treatment with L-NAME and aminoguanidine alone and in combination with low-dose onapristone on days 5-8 p.c. The animals were treated on days 5-8 p.c. analogously with experiment 3. The following groups were used (see Table 11 for numbers): group 1: vehicle control; group 2: onapristone alone; group 3: L-NAME alone; group 4: aminoguanidine alone; group 5: L-NAME plus onapristone; group 6: aminoguanidine plus onapristone.

Statistical analysis

The treatment effects on pregnancy rates were analysed using one-sided Fisher's exact test. For the statistical analysis of treatment effects on implantation numbers, the Wilcoxon test for comparison between the groups was applied.

Results

The effects of NO inhibition and low-dose onapristone after treatment during the preimplantation phase (days 1-4 p.c.)

The effects on pregnancy rates and implantation numbers after treatment on days 1-4 p.c (experiments 1 and 2)

Neither L-NAME nor aminoguanidine alone significantly affected pregnancy rates (Figure 2A and B) nor implantation numbers (Figure 2C and D) when administered before implantation. The implantation sites were normal in these groups (Figure 2; black bars). However, treatment with onapristone alone reduced the total number of implantation sites in both experiments by approximately 50–60% compared to controls (Figure 2C and D), the majority of them being macroscopically changed (smaller and in part haemorrhagic; presented as striped bars). In addition, the effects of each NOS inhibitor in combination with low-dose onapristone on both pregnancy rates and implantation numbers were quite dramatic. After L-NAME plus onapristone treatment, implantation sites were detectable in only two animals: six normal implantation sites in one animal and one very small implantation site in the second animal (Figure 2A and C). After aminoguanidine plus onapristone treatment, only two highly retarded and haemorrhagic implantation sites were observed in 1/7 animals (Figure 2B, D). Thus, the combination of onapristone and aminoguanidine completely prevented pregnancy.

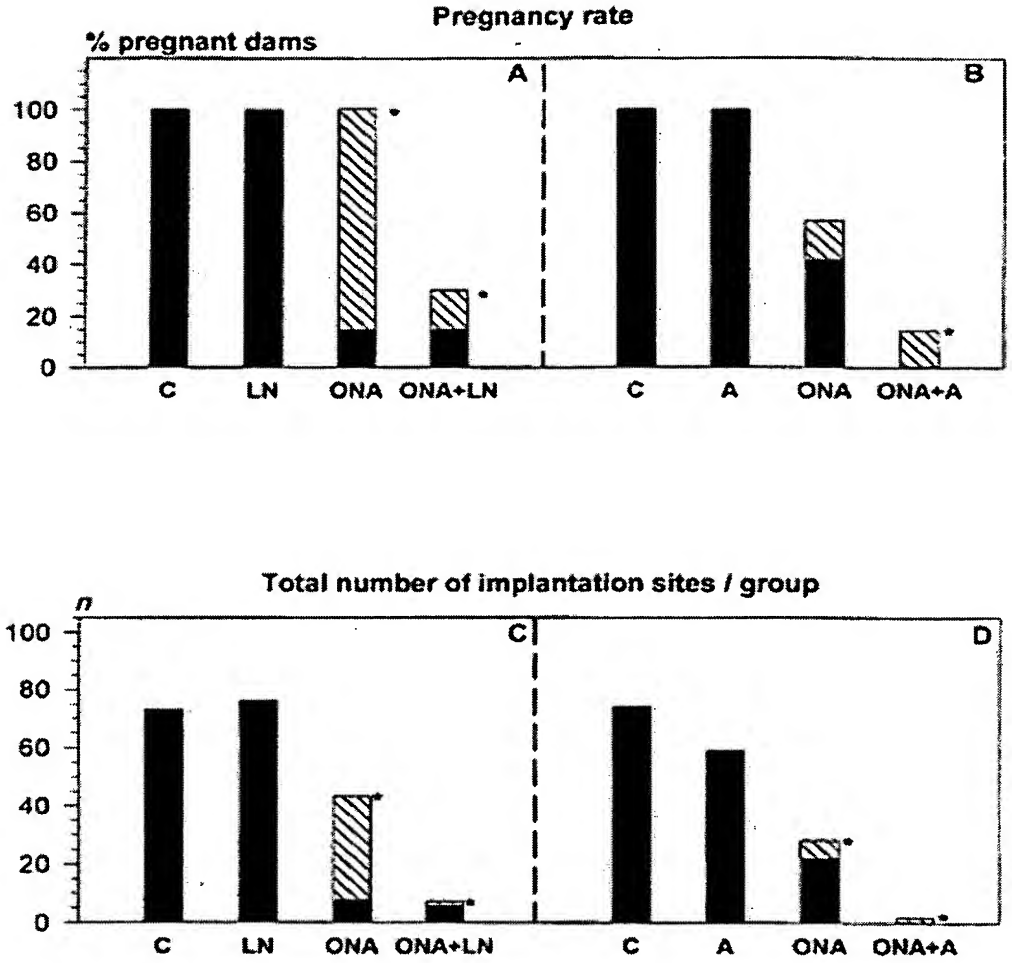
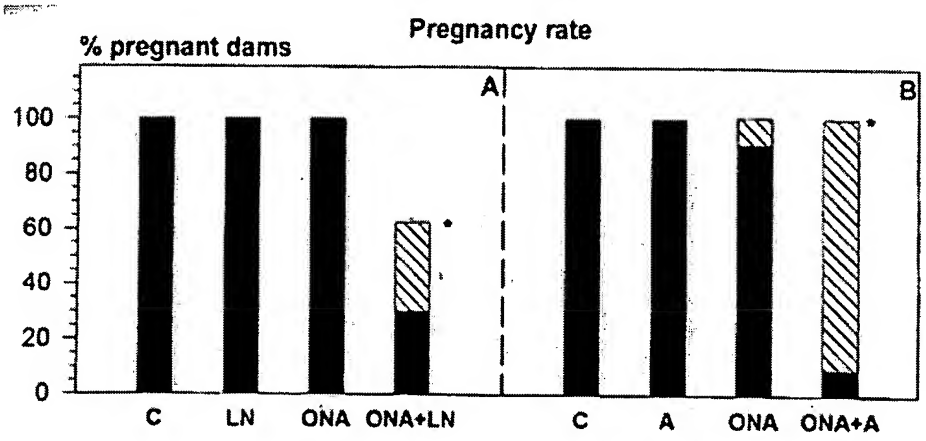


Figure 2. The effects of the NOS inhibitors L-NAME (LN) and aminoguanidine (A) in the presence or absence of low-dose onapristone (ONA) on pregnancy rates **(upper panel; A, B)** and implantation number (lower panel; B, C) established on day 9 p.c. after treatment at preimplantation (day 1-4 p.c.). There were seven animals per group. The autopsy was performed on day 9 p.c. Upper panel (A, B) presents the percentage of pregnant animals per experimental group. Black bars demonstrate the percentage of animals exhibiting macroscopically normal implantation sites. Striped bars present the percentage of animals showing macroscopically changed implantation sites (haemorrhagic and growth retarded). **Lower panel** (C, D) shows the total number of implantation sites per group. Black bars present the number of normal implantation sites, whereas striped bars demonstrate the number of macroscopically changed implantation sites. Asterisks* mark the statistically different values ($P < 0.05$) of normal implantation sites (black bars) from the respective control group.

The results above demonstrate, that while the percentage of animals exhibiting macroscopically normal implantation sites does not appear to be much different with the use of ONA versus ONA + LN (see black bars in panels A and C), the total number of implantation cites (see striped bars in the same panels) is significantly decreased, which does indicate that the two compounds exhibit a synergistic effect. With the use of ONA versus ONA + A, the results are even clearer as the number and percentage of normal

implantation cites completely disappears, and the total number and percentages also significantly decreases.

The effects of NO inhibition and low-dose onapristone after treatment during the pen - implantation phase (days 5-8 p.c.)The effects on pregnancy rates and implantation numbers after treatments on days 5-8 p.c. (experiments 5 and 6) Neither treatment alone had any significant effect on pregnancy rate (Figure 6A and B), nor on the number of implantation sites (Figure 6C and D) on day 9 p.c. Almost all of the implantation sites were macroscopically unchanged (presented as black bars in Figure 6). However, in combination with low-dose onapristone, both L-NAME and aminoguanidine significantly reduced pregnancy rates and the total number of implantation sites. Moreover, in those animals which remained pregnant, most implantation sites were macroscopically abnor-mal (necrotic and haemorrhagic areas, and small size; presented as striped bars in Figure 6). In animals treated with L-NAME plus onapristone, 23 normal versus 62 macroscopically abnormal implantation sites were recorded (Figure 6A and C). Similarly, after aminoguanidine plus onapristone treatment, only eight macroscopically normal implantation sites were discovered (Figure 6B and D).



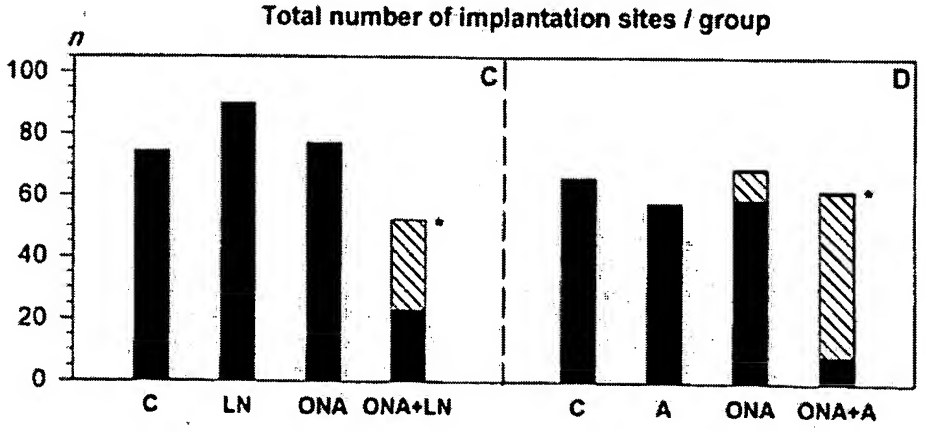


Figure 6. The effects of the NOS inhibitors L-NAME (LN) and aminoguanidine (A) in the presence or absence of low-dose onapristone (ONA) on pregnancy rates **(upper panel; A, B)** and implantation number **(lower panel; B,** C) established on day 9 p.c. after treatment at peri-implantation (days 5-8 p.c.). There were seven animals per group. Upper panel (A, B) presents the percentage of pregnant animals per experimental group. Black bars demonstrate the percentage of animals exhibiting macroscopically normal implantation sites. Striped bars present the percentage of animals showing macroscopically changed implantation sites (haemorrhagic and growth retarded). Lower panel (C, D) shows the total number of implantation sites per group. Black bars present the number of normal implantation sites, whereas striped bars demonstrate the number of macroscopically changed implantation sites. Asterisks* mark the statistically different values ($P < 0.05$) of normal implantation sites (black bars) from the respective control group.

The results above demonstrate, the significant decrease in normal implantation sites in all cases where a combination of ONA + LN and ONA + A were used versus the use of ONA alone.

The effects on pregnancy outcome (on day 19 p. c.) of treatment on days 1-4 p.c. (experiment 3) The results are summarized in Table I. No pregnancies were found after aminoguanidine plus onapristone treatment. After a combined L-NAME plus low-dose onapristone treatment there was a 50% inhibition of fertility and an increase in early resorptions. Only seven live, but extremely growth-retarded, fetuses were found in this group. There were no inhibitory effects on pregnancy rates after either treatment alone; however, onapristone treatment alone significantly reduced the number and weight of living fetuses.

Table I. Outcome of pregnancy on day 19 p.c. after treatment with the NOS inhibitors N^G-nitro-L-arginine methyl ester (L-NAME) and aminoguanidine (A) alone and in the presence and absence of low-dose onapristone (ONA) during the pre-receptive phase (days 1–4 p.c.; experiment 3). Asterisks mark the values which are significantly different from the vehicle control group ($P < 0.05$)

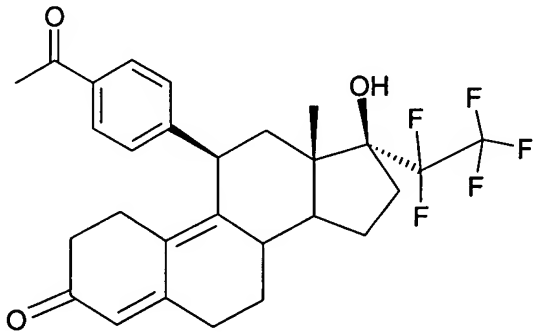
Group	Treatment	Total dams (*n*)	Dams pregnant (*n*)	Implantation sites (*n*)	Fetal weight (g) (mean ± SD)	Living fetuses (*n*)	Resorptions (*n*)
1	Vehicle	6	6	64	1.43 ± 0.16	60	4
2	ONA	7	7	53	1.06 ± 0.22*	44	9
3	L-NAME	7	7	67	1.43 ± 0.19	62	5
4	A	7	7	63	1.54 ± 0.15	57	6
5	L-NAME + ONA	6	3*	25*	0.76 ± 0.07*	7*	18*
6	A + ONA	7	0*	0*	-	0*	0

The effects on pregnancy outcome on day 19 p.c. after treatment on days 5-8 p.c. (experiment 7) Treatment with each NOS inhibitor alone did not produce any significant effects on the pregnancy rate nor on the number of living fetuses. A slight reduction in the number of living fetuses was observed after onapristone alone. Interestingly, both L-NAME and onapristone significantly reduced fetal weights when administered alone. However, both combination regimens exerted significant inhibitory effects on pregnancy rates and the number of living fetuses. The effects of L-NAME and onapristone were more pronounced than those of aminoguanidine plus onapristone (Table II).

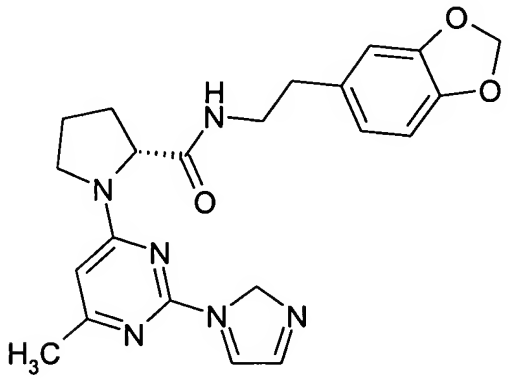
Table II. Outcome of pregnancy on day 19 p.c. after treatment with the NOS inhibitors N^G-nitro-L-arginine methyl ester (L-NAME) and aminoguanidine (A) alone and in the presence and absence of low-dose onapristone (ONA) during the peri-implantation phase on days (days 5–8 p.c.; experiment 7). Asterisks mark the values which are significantly different from the vehicle control group ($P < 0.05$)

Group	Treatment	Total dams (*n*)	Dams pregnant (*n*)	Implantation sites (*n*)	Fetal weight (g) (mean ± SD)	Living fetuses (*n*)	Resorptions (*n*)
1	Vehicle	7	7	70	62	1.37 ± 0.09	8
2	ONA	7	6	64	52	1.25 ± 0.01*	12
3	L-NAME	6	6	61	54	1.30 ± 0.11*	5
4	A	7	7	67	58	1.55 ± 0.19	9
5	L-NAME + ONA	7	2*	22*	19*	1.34 ± 0.01	3
6	A + ONA	7	4*	54	44	1.33 ± 0.10	10

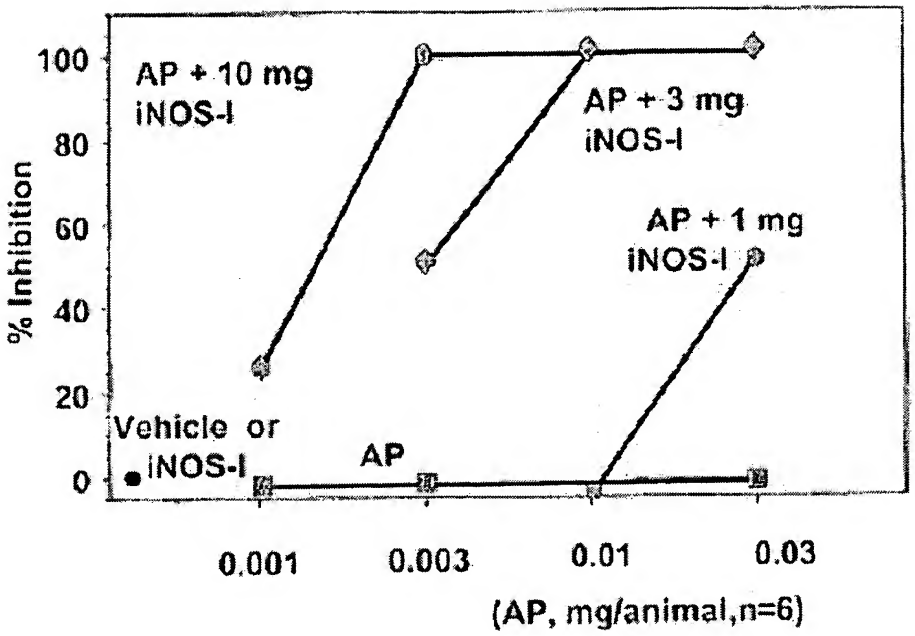
The next graph demonstrates the synergistic effect of an antiprogestin and an iNOS inhibitor whose structures are drawn below in inhibiting implantation in rats after p.o. treatment on days 2-5 p.c. on the same type of animals described above. The doses of the iNOS inhibitor are 1 to 10 mg/animal orally (p.o.) alone or in combination with the antiprogestin. The doses of antiprogestins are 0.001 to 0.03 mg/animal alone or in combination with the iNOS inhibitor. Both the iNOS inhibitor alone and the antiprogestin alone at any of these doses had no observable effect and had the same response as the vehicle. When both were administered together, a dose dependent inhibition of implantation was observed, which inhibition is much better than the additive effect of either the iNOS inhibitor alone or the antiprogestin alone, i.e., is synergistic.



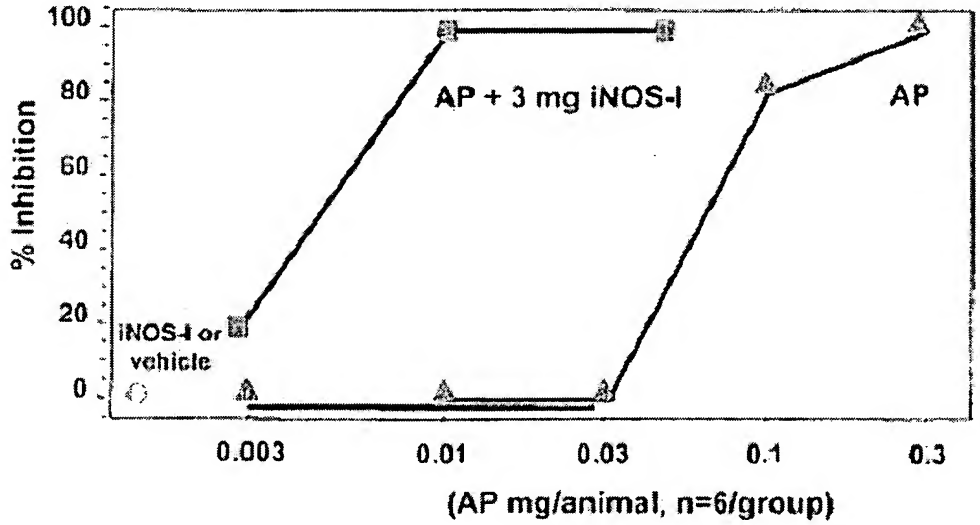
Antiprogestin



iNOS Inhibitor



The next graph demonstrates the synergistic effect of same the antiprogestin and the iNOS inhibitor used in the above graphs in inhibiting implantation in rats after p.o. treatment on days 2-5 p.c. on the same type of animals described above. The dose of the iNOS inhibitor is 3 mg/animal orally (p.o.) alone or in combination with the antiprogestin. The doses of antiprogestins are 0.003 to 0.3 mg/animal alone or in combination with the iNOS inhibitor. The iNOS inhibitor alone had no observable effect and had the same response as the vehicle. The antiprogestin alone had no observable effect below the dose of 0.03 mg/animal, and above such dose had a dose dependent effect. When both were administered together, a dose dependent inhibition of implantation was observed, which inhibition is much better than the additive effect of either the iNOS inhibitor alone or the antiprogestin alone, i.e., is synergistic.



Discussion

The results above in rats demonstrate the synergistic effect of an iNOS inhibitor and an antiprogestin, where substantial increase in the inhibitory effects of the antiprogestin is observed on pregnancy rates (see figures 2 A and B, 6 A and B, and Tables I and II) and on implantation (see figures 2 C and D, 6 C and D, and the last two graphs in this declaration). These effects are significant and are unexpectedly synergistic, since either treatment alone only marginally affected implantation and pregnancy outcome.

Nothing in the prior art could have led one of ordinary skill in the art to expect synergism of the combination of a NOS inhibitor and an antiprogestin.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Dec 22, 04
Date

Robert E Garfield
Dr. Robert E. Garfield